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Recycling and Uptake of Si(OH)_4 when Protozoan Grazers Feed on Diatoms

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Abstract

Herbivory of microzooplankton is an emerging key factor of diatom mortality in the ocean. As part of the microbial loop, protozoan grazers also feed on bacteria that accelerate the degradation of diatom detritus. The potentially pivotal effect of microzooplankton grazing on Si(OH)_4 recycling was investigated with cultures of single-celled diatoms, *Thalassiosira pseudonana* and *Chaetoceros gracilis*, and heterotrophic protozoans, the dinoflagellate *Oxyrrhis marina* and the ciliate *Strombidium* sp. Both grazers ingested diatoms and the bacteria in the non-axenic cultures. *C. gracilis*, whose frustule is “armed” with setae, was less suitable as a prey than *T. pseudonana*. Ingestion rates of *T. pseudonana* were comparable for *O. marina* and *Strombidium*, but the dinoflagellate produced two orders of magnitude more detrital bSiO_2 than the ciliate, due to the higher abundance reached by *O. marina*. Total net release of Si(OH)_4 was lower in the grazing treatments compared to the control possibly due to the reduced bacterial growth by microzooplankton bacterivory, and to the transient protection of detrital bSiO_2 in discarded feeding vacuoles. Over the first 24h, microzooplankton grazing even led to enhanced uptake of Si(OH)_4 by diatoms, confirming the potential of grazing to influence the silicification of diatom frustules. Subsequently however, the Si dynamics in bottles with grazers turned rapidly from net uptake to net Si(OH)_4 release. Protozoan grazers hence tie Si(OH)_4 recycling into the microbial loop by producing detrital bSiO_2 .

Key words

Diatoms, Microzooplankton, Silica recycling, *Thalassiosira pseudonana*, *Oxyrrhis marina*, Top-down effects

Running title

Si(OH)_4 Dynamics in Diatom-Grazer Interactions

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Introduction

The grazing activity of zooplankton plays a central role in plankton ecology and in the cycling of nutrients or trace elements in marine ecosystems (Barbeau et al. 1996; Barbeau et al. 2001; Hutchins and Bruland 1994; Lehman 1980; Twiss et al. 1996). Grazers stimulate the microbial loop and the attendant bacterial growth by releasing dissolved organic carbon (DOC) to seawater (Azam et al. 1983; Strom et al. 1997). In turn, protozoan grazers are important consumers of bacteria in the ocean (Jeong et al. 2008; Sherr and Sherr 1994) and there is increasing evidence that the grazing activity of such microzooplankton can keep phytoplankton growth in check (Archer et al. 2000; Calbet and Landry 2004; Irigoien et al. 2005).

Feeding strategies of microzooplankton on phytoplankton are diverse (Jacobsen 1999), yield high growth efficiencies (Tillmann 2004) and do not lead to the production of heavy fecal material as is the case for larger zooplankton. Mesozooplankton grazers such as copepods and euphausiids crush diatom cells during feeding, assimilate the carbon and condense the diatom debris in fast sinking fecal pellets (Cowie and Hedges 1996; Honjo and Roman 1978). Once aggregated, biogenic silica (bSiO_2) is protected from dissolution and exported to deeper layers (Moriceau et al. 2007; Schrader 1971; Schultes 2004). Unicellular protozoan grazers also feed on diatoms (Drebes and Schnepf 1998; Goldman et al. 1989; Naustvoll 2000) but either engulf their prey entirely or digest it externally discarding empty diatom frustules with the feeding vacuole. Diatom detritus emanating from protozoan grazing is susceptible to be degraded rapidly.

The recycling of Si(OH)_4 is influenced by both biotic and abiotic factors. Bacterial enzyme activity may accelerate the dissolution of diatoms by removing the protective coating from the frustule and exposing the silica walls to seawater (Bidle and Azam 1999; Bidle and Azam 2001). Dissolution is then driven by physical and chemical parameters such as temperature (Kamatani 1982) and ambient Si(OH)_4 concentrations (Greenwood et al. 2001; Truesdale et al. 2005).

The rate of bSiO_2 dissolution is crucial in alleviating growth limitation of diatoms when upwelling of nutrient rich waters does not provide sufficient Si(OH)_4 for new primary production as appears to be the case in the equatorial Pacific (Dugdale and Wilkerson 1998; Leyneart et al. 2001). Diatoms from tropical waters have low cellular Si:C ratios compared to highly silicified diatoms encountered in the Southern Ocean (Brezinski 1985). Their high Si content is believed to result from reduced growth rates under iron limitation (Hutchins and Bruland 1998) or light stress (Claquin et al. 2002) and from a defense strategy against zooplankton grazing (Smetacek 2001). Also morphological diversity in diatoms, such as setae extruding from the frustules or the formation of colonies, are thought to protect the cells from predation (Verity and Smetacek 1996).

Recently, Sherr and Sherr (2007) concluded that, in addition to ciliates, heterotrophic dinoflagellates represent an important source of diatom mortality. The role of microzooplankton grazing in Si(OH)_4 recycling is however pivotal. Grazing and digestion of diatoms may accelerate bSiO_2 dissolution, grazing on bacteria slow it down. Both processes are likely to occur on similar time scales. The objective of this experimental study is to explore the effect of microzooplankton grazing on changes in the availability of silicic acid with grazing experiments in non-axenic diatoms cultures. The working

hypothesis is that microzooplankton grazing will increase the release of Si(OH)_4 from the diatom debris over the timecourse of the incubation.

Results

Changes in Cell Abundance

A representative example of the timecourse of changes in diatom abundance in the control, and diatom and microzooplankton abundance in the grazing bottles is presented in Figure 1a. For changes in the abundance of bacteria an example is presented in Figure 1b. Grazing on diatoms by microzooplankton occurred in all experiments, except when *Chaetoceros gracilis* was fed to the ciliate *Strombidium* sp. Bacterial growth was generally reduced in the grazing bottles compared to the control. In the following, results on trophic dynamics will be presented for each experiment in terms of the calculated growth rates and grazing rates plotted for every time interval sampled. Si(OH)_4 dynamics for each incubation are presented accordingly.

Dinoflagellate Grazing Experiments

Oxyrrhis marina Feeding on *Thalassiosira pseudonana* (Experiment 1)

Immediately after addition of the grazer *Oxyrrhis marina*, the dinoflagellate began ingesting *Thalassiosira pseudonana* at an average rate of 27 ± 20 diatom cells per grazer per day (Fig. 2a). The ingestion led to immediate growth of the dinoflagellate during the first 24h, at a growth rate greater than 1.5 per day (Fig. 2b). On the second day of the experiment, the grazing activity on diatoms was reduced by a factor of 2 and no significant growth for *O. marina* could be determined. After 48h of incubation and onwards, *O. marina* did not appear to be grazing diatoms and the dinoflagellate population slightly declined.

Bacterial growth was significantly reduced (Friedman and Tukey post-hoc test; Table 2) in the grazing bottles compared to the control bottles (Fig. 2c). Both treatments showed growth of bacteria during the first 24h, whereas on the second day bacteria in the grazing incubations began to decline. The decline in bacterial numbers after 48h was significantly higher in the control than in the bottles with added grazers.

Net input of Si(OH)_4 from the diatoms over the entire course of the experiment was $3.5 \pm 0.2 \mu\text{mol l}^{-1}$ in the control compared to $2.1 \pm 0.3 \mu\text{mol l}^{-1}$ in the grazing treatment (Table 3). Relative changes in the pool of Si(OH)_4 in the three time intervals were significantly different between the treatments (Friedman $\chi^2 = 14.18$; $p = 0.01$). Control and grazing bottles gradually turned from the removal of Si(OH)_4 to accumulation (Figure 2d). During the first 24h, both treatments showed net removal which was significantly faster (Mann-Whitney $p = 0.04$, Table 4) in the grazing bottles ($-43 \text{ nmol Si(OH)}_4 \text{ l}^{-1} \text{ d}^{-1}$) compared to the control ($-400 \text{ nmol Si(OH)}_4 \text{ l}^{-1} \text{ d}^{-1}$). Over the course of the second day, some Si(OH)_4 still disappeared from the control, whereas the grazing bottles started to release Si(OH)_4 . From 48h onwards, Si(OH)_4 concentrations increased in all bottles, with significantly lower values observed in the grazing treatment.

Oxyrrhis marina Feeding on *Thalassiosira pseudonana* (Experiment 2)

The experiment with the dinoflagellate feeding on *T. pseudonana* was repeated and results for the grazing and growth dynamics of *O. marina* (Figs. 3a, b) were very similar to those observed in the previous experiment. Differences are noted for the bacterial growth, which is again reduced in the grazing bottles compared to the control (Fig. 3c) but in experiment 2 bacteria continued to grow in all bottles over the entire duration of the incubation. Bacterial numbers at time zero were an order of magnitude lower in experiment 2 compared to experiment 1.

Si dynamics were again significantly different in control and grazing incubations (Friedman $\chi^2 = 13.62$; $p = 0.02$; Table 2). Total release of Si(OH)_4 over the entire course of the incubation was higher in the control than in the grazing treatment. A net amount of $0.3 \pm 0.0 \mu\text{mol l}^{-1}$ accumulated in the control compared to a net removal of $-1.3 \pm 0.0 \mu\text{mol l}^{-1}$ observed in the grazing treatment (Table 3). Si(OH)_4 disappeared from the Si(OH)_4 pool significantly faster in the grazing treatment during the first 24h (Mann-Whitney $p = 0.04$, Table 4), whereas the control showed a slight release of Si(OH)_4 into solution (Fig. 3d). Comparable Si dynamics for both experiments are also observed on day 2 when net removal of Si(OH)_4 occurred in the control bottles but net release was observed in the grazing bottles. Although the Tukey post-hoc test does not indicate a significant difference, this net release over the last two days was apparently higher in the grazing incubation compared to the control where the Si(OH)_4 pool remained unchanged (Fig. 3d).

Oxyrrhis marina Feeding on *Chaetoceros gracilis* (Experiment 3)

In contrast to the two previous experiments, grazing of the dinoflagellate on *Chaetoceros gracilis* increased, rising from 4 cells per grazer per day during the first 24h to 12 cells per grazer per day from 48h onwards (Fig. 4a). Maximum ingestion rates for *C. gracilis* were by a factor of 4 lower than those determined for *Thalassiosira pseudonana* (Figs. 2a, 3a). Moderate and variable growth of the dinoflagellate was observed during the first day and increased to a maximum of 0.7 per day during the second day (Fig. 4b), slightly lower than the growth rates obtained on a diet of *T. pseudonana* (Figs. 2c, 3c).

Bacteria continuously grew in all incubation flasks with a gradual reduction in growth rate from 0.8 to 0.1 per day in the control and from 0.5 to 0.1 per day in the grazing bottles (Fig. 4c). Significantly lower bacterial growth in the grazing treatment can be determined for day 1 (Tukey post-hoc test, Table 2).

Again, net removal of Si(OH)_4 from solution was observed during the first 24h in the control and the grazing bottles (Fig. 4d) but no statistical difference was determined between treatments during this time interval (Table 4). For the entire duration of the experiment, however, the Friedman test revealed significantly different Si dynamics among treatments ($\chi^2 = 14.0$; $p = 0.01$). During the second day, low increase of Si(OH)_4 was measured in the control versus net removal of Si(OH)_4 in the incubations with added *O. marina*. Finally, the system shifted to a clear release of Si(OH)_4 from 48h onwards, at similar rates in control and grazing incubations. The total net accumulation of Si(OH)_4 in the control was $0.7 \pm 0.2 \mu\text{mol l}^{-1}$. Contrary to the two experiments where *O. marina* was feeding on *T. pseudonana*, significantly (Mann-Whitney $p=0.04$) higher net accumulation of $1.7 \pm 0.3 \mu\text{mol l}^{-1}$ was observed in the grazing treatment (Table 3).

Ciliate Grazing Experiments

Strombidium sp. Feeding on *Thalassiosira pseudonana* (Experiment 4)

The ciliate *Strombidium* sp. fed on *Thalassiosira pseudonana* with ingestion rates comparable to those determined for the dinoflagellate. Ingestion of 12 to 25 diatom cells per grazer per day was determined during the entire incubation period (Fig. 5a). Ciliate populations declined during days 1 and 2 before starting to grow from 48h onwards (Fig. 5b).

Bacterial populations declined in all incubation flasks, with the exception of some growth that occurred during the first 24h in the control bottles (Fig. 5c). On the last two days, bacterial numbers declined significantly more quickly in the control compared to the incubations containing ciliates (Tukey post-hoc test, Table 2), comparable to the observations made in experiment 1 where the dinoflagellate fed on *T. pseudonana* (experiment 1).

Also changes in Si(OH)_4 concentrations were similar to the results obtained in experiment 1. According to results from the Friedman test, overall Si dynamics in this experiment were not significantly different between treatments ($\chi^2 = 10.72$; $p = 0.06$). Yet, a significantly higher removal of Si(OH)_4 from the Si(OH)_4 pool during the first 24h (Mann-Whitney $p = 0.04$, Table 4) could be measured in the grazing treatment compared to the control (Fig. 5d). During the second day, slight net removal of Si(OH)_4 was still detectable in the control but the grazing treatment started to release Si(OH)_4 to solution. From 48h onwards the system also shifted to Si(OH)_4 release in the control, but no significant difference (Tukey post-hoc test) could be determined between the treatments.

Over the entire duration of the experiment, net changes in Si(OH)_4 concentrations were balanced in the control ($-0.1 \pm 0.2 \mu\text{mol l}^{-1}$) and some net removal of $-0.4 \pm 0.1 \mu\text{mol l}^{-1}$ was determined in the grazing treatment (Table 3).

Strombidium sp. Feeding on *Chaetoceros gracilis* (Experiment 5)

In general, grazing and growth of *Strombidium* sp. were very low when the ciliate was added to a culture of *Chaetoceros gracilis*. Diatom ingestion by the ciliate could be calculated only for the second day with an average of 2 cells per grazer per day (Fig. 6a). Nevertheless, low and very variable growth rates with maximum values of 0.2 per day were observed for the ciliate during the first two days (Fig. 6b).

In all incubations, bacteria grew continuously (Fig. 6c). Bacterial growth was enhanced in both treatments on the second day, without significant difference (Tukey post-hoc test) between control and grazing incubations.

As was observed in the experiment where the ciliate grazed on *Thalassiosira pseudonana*, Si dynamics were not statistically different between control and grazing treatments (Friedman $\chi^2 = 4.85$; $p = 0.43$). Net removal of Si(OH)_4 from the Si(OH)_4 pool was observed during the first 24h at without statistical difference among the treatments (Fig. 6d), comparable to what has been observed when the dinoflagellate fed on *C. gracilis*. During the second day, Si(OH)_4 accumulated in both treatments, again without significant difference between the two (Tukey post-hoc test, Table 2). In the final phase of the experiment, slight Si(OH)_4 net removal from solution was again observed in the control, compared to some release of Si(OH)_4 in the grazing treatment, but it has to be

acknowledged that changes in Si(OH)_4 concentrations determined in this time interval are close to detection limit and should be considered as constant.

Total change of Si(OH)_4 was without significant difference among treatments (Mann-Whitney $p = 0.3$; Table 3), with virtually unchanged concentrations in the control ($-0.2 \pm 0.1 \mu\text{mol l}^{-1}$) and the grazing treatment ($-0.1 \pm 0.3 \mu\text{mol l}^{-1}$).

Discussion

In this experimental study, non-axenic cultures of two small, centric and single celled diatoms were exposed to grazing by a dinoflagellate and a ciliate. Changes of Si(OH)_4 concentrations were monitored during the incubations. Compared to nitrate, phosphate and ammonia (e.g. Andersen et al. 1991; Goldman et al. 1985; Johannes 1965; Lehman 1980) very little attention has been given so far to the question of how trophic interactions of herbivorous grazers and diatoms change Si dynamics. Si(OH)_4 differs from the other macro- and micronutrients in that it is required by diatoms but not taken up and accumulated by zooplankton or bacteria. Any increase observed in the Si(OH)_4 concentrations during the incubation was presumably due to dissolution of diatoms grazed by the microzooplankton and/or degraded by the bacterial assemblage in the batch cultures.

Grazing Success of Microzooplankton on Diatoms

Differences in the ingestion rates of diatoms and the growth response of the microzooplankton could be observed among the four predator-prey combinations. This lends support the notion that not all diatoms are equally suitable as prey for protozoan grazers (Naustvoll 2000; Verity and Villareal 1986). *Thalassiosira pseudonana* was generally ingested at higher rates than *Chaetoceros gracilis*, by both predators. The grazing response of *O. marina* when feeding on *T. pseudonana* strongly resembled the dynamics described by Öpik and Flynn (1989), with highest ingestion rates occurring over the first 24h of the incubation. In comparison, *C. gracilis* was ingested with a temporal delay and at reduced rates. The siliceous setae of *C. gracilis* were clearly visible under the microscope in samples from time zero but were not observed after 24h when ingestion by *O. marina* started to increase. As it appears, once the setae dissolved or broke, *C. gracilis* became vulnerable to predation by *O. marina*. Virtually no ingestion of *C. gracilis* by the ciliate *Strombidium* could be detected, but the ciliate ingested *T. pseudonana* at rates comparable to those observed for the dinoflagellate. These results point to a low suitability of *C. gracilis* as prey, potentially due to the setae on the frustule as defense against protistan grazing (Verity and Villareal 1986).

The degree and timing of nutrient recycling may be affected by differences in the ingestion and growth response of the grazer with respect to its prey. Feeding on *Thalassiosira pseudonana* supported growth of both microzooplankton grazers at rates consistent with previously obtained results (Goldman et al. 1989; Jeong et al. 2003; Strom and Morello 1998). Growth of *Oxyrrhis marina* rapidly followed ingestion within 12 to 24h. Highest growth rates were hence observed on the first day when *O. marina* was feeding on *T. pseudonana*, and growth was consequently delayed when *C. gracilis* was offered as prey. *Strombidium* sp. grew with a 48h delay despite continuous ingestion of *T. pseudonana* (experiment 4). Mortality of ciliates in the acclimation phase of 48h has been reported (Montagnes 1996 and references therein) and may explain the initial

decrease in ciliate abundance observed in experiment 4, although it is unclear then why no mortality occurred when the ciliate was added to the culture of *C. gracilis* (experiment 5, Fig. 6b). Digestion of prey in the food vacuoles of microzooplankton is thought to be rapid (e.g. 75 min in the study of Dolan and Šimek 1997), but fusion of several food vacuoles can prolong digestion and delay expulsion of undigested matter. First mini-pellets, i.e. discarded feeding vacuoles with frustules surrounded by a thin membrane, started to appear 6h after the start of the experiment (microscopic observation) when *O. marina* grazed on *T. pseudonana* but were not observed in experiments with *Strombidium* sp. These results indicate that digestion and assimilation of diatom carbon, and possible recycling of bSiO₂ was very rapid for the *O. marina* compared to the *Strombidium* sp..

Even more important though, the amount of bSiO₂ that was grazed and transferred to the detrital pool was strikingly different among predator-prey combinations (Table 3). Based on ingestion rates of diatoms, the oligotrich ciliate processed less than 1 % of the diatom cells, whereas the dinoflagellate ingested and digested 4 % of the cells in the experiment with *C. gracilis* and over 70 % of the bSiO₂ when grazing on *T. pseudonana*. As has been discussed above, *C. gracilis* appeared to be unsuitable as prey and consequently the lower ingestion rate led to lower detrital bSiO₂ production from *C. gracilis*. In comparison, *T. pseudonana* was ingested at similar rates by both protozoans (Table 4). The observed differences in detrital bSiO₂ production from *T. pseudonana* therefore arise from the respective predator concentrations in the experiments. The one order of magnitude higher abundance of *O. marina* in the experiments (Table 1) reflects the higher concentrations that this grazer reached in the stock cultures (see Methods). Abundances of heterotrophic dinoflagellates and ciliates in the field range from less than ten to several thousand cells ml⁻¹ during microzooplankton “blooms” when top-down control by metazoan zooplankton is reduced (Jeong 1999; Smetacek 1981; Tillmann 2004). Our results are therefore indicative of the large differences that can be expected in the grazing impact of protozoan grazers, depending on the diatom prey species and protozoan grazer abundance.

Growth Dynamics of Bacteria in the Experiments

Grazing on bacteria appears to be important for both *Oxyrrhis marina* and *Strombidium* sp. The presence of grazers led to significantly different bacterial growth in all experiments (Table 2). Bacterial growth rates in the grazing bottles were lower than in the control in four of the five incubations. When bacteria started to decline in the control incubations of experiment 1 and 4, however, this decline was less pronounced in the grazing bottles (Figs. 2c, 5c). Probably, the production of DOC by the microzooplankton (Strom et al. 1997) continued to fuel bacterial growth. Strom et al. observed a general increase in bacterial growth rate in the presence of grazers, except when *O. marina* was added, who is known to ingest bacteria (Jeong et al. 2008). We therefore conclude that grazing losses of bacteria to *Strombidium* and *O. marina* in our study generally outweighed growth stimulation via DOC release in the early stages of the incubation. During the last time interval, however, grazing acted to sustain actively growing bacterial populations that can colonize and degrade diatom detritus.

Dissolved Silicate Dynamics under Varying Trophic Interactions

Three processes are likely to govern Si dynamics in the experimental system: the magnitude of ingestion of diatoms, indicating the amount of bSiO_2 removed from the suspension, the time of growth of microzooplankton representative of the complete digestion of the ingested diatoms, and potential grazing or substrate limitation of bacterial activity. Also for Si dynamics, three general patterns can be observed: net removal, balanced systems and net release to solution.

Net Release of Si(OH)_4 to Solution

Highest release rates of Si(OH)_4 in the system were generally observed in the third time interval (Figs. 2d, 3d, 4d, 5d) after 48h of incubation. At this point in time, most heterotrophic activity, i.e. bacterial growth, microzooplankton grazing and microzooplankton growth, has already peaked and it can be assumed that release of Si(OH)_4 from bSiO_2 dissolution was then governed by chemical processes. Release rates of Si(OH)_4 were not distinctively higher or faster in the grazing compared to the control incubations indicating that chemical dissolution rates were not influenced by the number of trophic levels.

The working hypothesis of this study stated that total net release of Si(OH)_4 would be higher in grazing treatments due to the ingestion and digestion of diatoms by the microzooplankton. This hypothesis (H_0 : $\text{Si}_{\text{grazing}} = \text{Si}_{\text{control}}$; H_1 : $\text{Si}_{\text{grazing}} > \text{Si}_{\text{control}}$) was tested with Mann-Whitney statistics, and H_1 was rejected for 4 out of 5 experiments (Table 3). In experiments where over 70% of the *Thalassiosira pseudonana* cells had been grazed by *Oxyrrhis marina*, on average $1.5 \mu\text{mol l}^{-1}$ less Si(OH)_4 accumulated in the grazing treatment compared to the control.

Dissolution may have been slowed down by the fact that diatom detritus left by microzooplankton is aggregated in so called mini-pellets. Empty diatom frustules inside feeding vacuoles were only observed in the experiments with *O. marina* and *T. pseudonana*, supporting our hypothesis. The effect of microzooplankton grazing would hence be comparable to that of mesozooplankton grazing (Schultes 2004). In similar experiments with copepods and krill, bSiO_2 was protected from dissolution in fecal material, which remained intact for several months at 4°C . The discarded feeding vacuoles of microzooplankton, however, are likely to protect the diatom frustules for considerably shorter time spans. At 15°C , Stoecker (1984) reported disintegration of ciliate feeding vacuoles after a maximum of 6 days. Protozoan fecal pellets appear to be robust enough to allow their occasional observation in sediment traps (Buck and Newton 1995; Nöthig and von Bodungen 1989), but depending on the ambient temperature (Bidle et al. 2002), settling rates of the mini-pellets and depth of the mixed layer, detrital bSiO_2 from microzooplankton grazing will most probably be recycled by bacteria in the surface layer.

Balanced Si Dynamics

Stable Si(OH)_4 dynamics in the experimental system indicate either no recycling due to low biological activity, or a tight coupling of processes driving recycling and uptake of Si(OH)_4 . Results of experiment 5, in which no significant differences can be detected in Si dynamics between treatments are indicative of the first possibility. The ciliate did not

ingest diatoms, and bacterial growth was comparable in control and grazing bottles. Since no dissolution is observed in either of the treatments despite highest diatom cell numbers of all experiments (Table 1), substrates other than carbon associated with biogenic silica must have sustained bacterial metabolism.

Net Removal of Si(OH)_4

A net removal of Si(OH)_4 from the dissolved phase can be determined in all five grazing experiments over the first 24h time interval. Unfortunately, only for experiment 2 bSiO₂ concentrations are available to verify whether this loss can actually be attributed to uptake of Si(OH)_4 by diatoms. The decrease of Si(OH)_4 concentrations by 2.2 μmol Si(OH)_4 in the grazing bottles (Fig. 3d) is indeed reflected by an increase in bSiO₂ concentrations by 3.7 μmol (Fig. 7). The precision of the alkaline digestion method is close to 10%, compared to 2 % for the determination of Si(OH)_4 on the auto-analyzer (Ragueneau et al 2005). Considering bSiO₂ background concentrations of 17 $\mu\text{mol l}^{-1}$ in experiment 2, the difference between Si(OH)_4 loss and bSiO₂ uptake falls within the analytical error of bSiO₂ measurements. It can be assumed with some confidence that the increase in bSiO₂ is representative of the loss of Si(OH)_4 from the solution.

When *Thalassiosira pseudonana* is offered as a prey to either the dinoflagellate or the ciliate, uptake of Si(OH)_4 is generally higher in grazing treatments than in the control. This is not the case for incubations with *Chaetoceros gracilis*. Similar differences among diatom species have been observed in grazing experiments conducted during May and June 2000 (S.Schultes unpubl. data; results included in Table 4). The copepod *Acartia clausii* was added to cultures of *Thalassiosira weissflogii* (8 μm) and *Thalassiosira punctigera* (60 μm) and allowed to feed for 24h in the dark. In both incubation experiments, net uptake of Si(OH)_4 was observed. Uptake was similar in the control and grazing treatment when *A. clausii* fed on *T. punctigera*, but differed by a factor of five among treatments when *T. weissflogii* was offered as prey. Potentially, the ingestion rate with which the grazers feed on the respective diatoms plays a role in the stimulated uptake. Ingestion rates of *A. clausii* for *T. weissflogii* were 1309 ± 223 cells per grazer per day compared to 42 cells ± 24 per grazer per day for *T. punctigera* (Jansen 2002). In this study, ingestion rates for *T. pseudonana* were on average 30 cells per grazer and day, compared to 4 cells per grazer and day for *C. gracilis* (Table 4). The significantly higher Si(OH)_4 uptake hence occurred in the grazing flasks with higher ingestion rates.

The apparent stimulation of uptake of Si(OH)_4 of diatoms under grazing pressure in dark incubations lends support to the hypothesis that high silicification is a protection against grazing (Smetacek 2001). Higher Si:C ratios of diatoms grown in media which previously contained grazing copepods have been reported (Pondaven et al. 2007). The exact mechanism however, remains to be discovered, and the study of Pondaven et al. (2007) indicates that it may not be the active ingestion but some chemical cue left by the grazers leading to higher bSiO₂ per cell quotas. In this study, uptake of Si(OH)_4 is stronger in grazing flasks but also detected in the control for the first 24h. The diatom batch cultures were taken from the culture room in the early light phase of the light:dark cycle, the experiment set up and all incubation containers put to obscurity for the grazing incubation. Uptake of Si(OH)_4 can occur in the dark since energy for silicification is linked to respiration rather than photosynthesis (reviewed in Martin-Jezequel et al. 2000). Granum and Mykkestad (1999) demonstrated how the addition of NH_4^+ to diatoms placed

in the dark activates respiration based on glucan. It is therefore conceivable that the NH_4^+ excretion of grazers has the potential to interfere with silicification of the diatom (Fig. 8) by activating diatom respiration and Si(OH)_4 uptake. Future work should try to unravel if the energetic specificity of silicon metabolism in diatoms combined with zooplankton grazing activity which usually peaks during dusk and dawn are at the physiological origin of the watery arms race (Smetacek 2001).

Pivotal Role of Microzooplankton Grazing

The relative importance of microzooplankton and bacteria in Si(OH)_4 recycling may be appreciated from our data but quantitatively the amount of released Si(OH)_4 cannot be clearly attributed to either of the two heterotrophic components present in the grazing treatments. The crucial role of grazers is most probably not the direct dissolution of bSiO_2 during digestion since the pH in feeding vacuoles tends to be strongly acidic (Fok et al. 1982) and bSiO_2 dissolves under alkaline conditions (Greenwood et al. 2001). Bacteria hasten the dissolution of diatom detritus and have probably been responsible for most of the Si(OH)_4 release observed in this study. Assuming maximum bSiO_2 concentrations of $30 \mu\text{mol l}^{-1}$ - of which 70% can be considered detrital when the dinoflagellate grazed on *Thalassiosira pseudonana* - and a turnover rate of 8% per day (table 2 in Bidle and Azam 1999) the increase of Si(OH)_4 in our experiments can be quantitatively attributed to bacterial activity.

A qualitative interpretation of the results points to an accelerating role played by the micrograzers in providing detrital bSiO_2 and allowing nutrients to be fed back into diatom production. During the first two days of the dark incubation microzooplankton grazing produced most detrital bSiO_2 but also reduced bacterial growth. Bacteria-mediated dissolution of the fresh diatom detritus may therefore have been diminished by microzooplankton bacterivory during the first 24h. If any dissolution occurred, the Si(OH)_4 was immediately taken up again and could not be measured with our analytical approach.

A decoupling of Si dynamics can usually be observed on the second day of the incubation where control and grazing treatments display opposite Si dynamics i.e. either net removal or net release to solution. In all experiments with the diatom *Thalassiosira pseudonana*, the system in grazing bottles switches from strong uptake to release within 24 h. In experiment 3, where the ingestion of *Chaetoceros gracilis* by *Oxyrrhis marina* was delayed, the switch takes place with equal delay, i.e. between the second and third time interval. These observations indicate that when diatoms are ingested by microzooplankton grazers, the system quickly starts to release Si(OH)_4 . The importance of microzooplankton hence consists in efficiently feeding on diatoms and producing easily accessible and light weighed detritus, which can then be hydrolyzed by bacteria in the microbial loop (Fig. 8). Field observations of stable and low, ambient Si(OH)_4 concentrations despite strong bSiO_2 production and dissolution events (Beucher et al. 2004) exemplify the rapid interplay of microzooplankton and bacteria in regulating Si(OH)_4 availability. Further research will require an isotopic approach (for example Fripiat et al. 2009) to quantify the role of both heterotrophic compartments and provide rates for biogeochemical models.

Our findings place the grazing activity of heterotrophic protozoans on diatoms in the center of attention for future research on Si(OH)_4 recycling in pelagic systems (Fig. 8).

Protozoan herbivory will lead to fast recycling and sustain regenerated diatom growth in areas where primary production is Si-limited. In comparison, mesozooplankton grazing on diatoms or protozoans will extract C and Si from this regenerative system (Wassmann 1998). Results further demonstrate that the effect of protozoan grazers on Si(OH)_4 dynamics varies depending on the grazer and diatom species who are engaged in the trophic interaction. Finally, this study adds evidence to the notion that top-down effects can influence the growth dynamics of diatoms and the structure of pelagic ecosystems (Verity and Smetacek 1996).

Methods

Incubation Experiments and Culture Conditions: Five grazing experiments were conducted during 2006 and 2007. Representative of two major taxonomic groups in aquatic systems, the heterotrophic dinoflagellate *Oxyrrhis marina* (SAG strain B21.89; “Sammlung von Algenkulturen, Universität Göttingen”), and an oligotrich ciliate of the genus *Strombidium* sp. (isolated from the Bay of Brest) were chosen as grazer models and added to late exponential or early stationary phase cultures of *Thalassiosira pseudonana* (CCMP strain 1015) and *Chaetoceros gracilis* (obtained from the IFREMER aquaculture station in Argenton, France). Both prey diatoms are single-celled and of small size (approx. 5 μm). The setae on *C. gracilis* cells represent a major morphological difference between both diatoms which may affect prey suitability and ocean biogeochemistry (*sensu* Smetacek et al. 2004; Verity and Smetacek 1996).

Control and grazing treatments were incubated in triplicate, in 500ml polycarbonate bottles, at 20°C and in the dark for up to 7 days. Sub-sampling took place after 24h, 48h, and 96h or 168h respectively. Details on experimental conditions are presented in Table 1. Variations in the respective initial abundance of predator, prey and bacteria in the set-up reflect differences in the growth of diatoms, grazers and bacteria previous to the start of the experiment. It has been tested whether the initial abundance of diatoms, grazers or bacteria influenced final net changes in Si(OH)_4 concentration but no correlation (Pearson, bilateral test, $p < 0.05$) between the parameters could be detected.

All phytoplankton cultures were grown as batch cultures under a 16h light: 8h dark cycle, in f/2 medium (Guillard and Ryther 1962). Dissolved silicate concentrations in the f/2 medium were, however, reduced to 30 μM . In preliminary incubations, both diatoms used in this study showed similar initial exponential growth rates at 100 μM (i.e. the full Si(OH)_4 addition of the f/2 medium) and at 10 μM , but reached lower cell densities in the 10 μM treatment (data not shown). An addition of 30 μM Si(OH)_4 to the cultures in the final experimental set-up allowed to reach sufficient diatom cell densities but kept the Si(OH)_4 background concentrations low ($< 5 \mu\text{M}$; see Table 1) in order to measure differences in the temporal evolution of Si(OH)_4 concentrations between experimental treatments.

Microzooplankton cultures were reared on batch cultures of the chlorophyte *Dunaliella salina*, grown in f/2 medium without silicate addition. Stock cultures reached densities of 5000 cells ml^{-1} (*Oxyrrhis marina*), and 400 cells ml^{-1} (*Strombidium* sp.). These concentrations are comparable to those reached in other studies (Dolan and Šimek 1997; Jeong et al. 2003). Protozoans were not fed in the last 48h before the start of the experiment. This assured minimal transfer of *D. salina* into the grazing treatments.

Chemical Analysis of Si: For the determination of silicic acid (Si(OH)_4) a 20-50 ml sub-sample of the experimental volume was filtered through a 0.6 μm polycarbonate membrane and the filtrate was analyzed on a Technicon Autoanalyser according to the method of Tréguer and Le Corre (1975). The filter was frozen at -20°C for the later determination of biogenic silica (bSiO_2) following the protocol proposed by Ragueneau and Tréguer (1994). For the alkaline digestion of bSiO_2 the filter is resuspended in 4 ml NaOH (0.2 M), digested for 40 min at 100°C , neutralized with 1 ml HCl (1 M) and Si(OH)_4 concentrations determined accordingly.

Cell Abundance of Bacteria, Diatoms, Dinoflagellate and Ciliate: Abundances of the ciliate were estimated from samples fixed with Lugol's solution and counted on an inverted microscope (Utermöhl 1958).

Cell abundance of bacteria, diatoms and the dinoflagellate were determined on sub-samples fixed with glutaraldehyde (0.5 % final concentration) and analyzed by flow cytometry. Methods were adapted from routine protocols (Marie et al. 1997; Marie et al. 1999) developed and used at the neighboring marine station in Roscoff (France) to estimate plankton and bacterial abundances in sea-water by flow cytometry. The protocol used in this study was established in our laboratory for bivalve cells analysis (Delaporte et al. 2003; Lambert et al. 2003; Lambert et al. 2007). Preliminary experiments were performed in order to test flow cytometry reproducibility and to compare diatom counting obtained by microscopy to flow cytometry analysis. As a result, repeated analysis ($n=14$) of the same sample by flow cytometry showed a very low variation coefficient of 1.32%. Comparison of diatom counts obtained with microscopy and flow cytometry (5 sampling dates x 3 replicates) showed no significant differences in the standard deviation for each sampling date (F-test, 95% confidence level) and no difference in the mean value of four out of five sampling dates (t-test, 95% confidence level). Also for *O. marina*, mean cell abundance estimated with the microscope and with flow cytometry were not significantly different over the time course of the experiment (t-test, 95% confidence level). Around 1 ml of each individual fixed sample was filtered through an 80 μm -mesh and transferred into two separate tubes for flow cytometry (12x75 mm, 5 ml polystyrene round bottom test tube, BD Falcon™ ref 352052). The first one was analyzed without delay to estimate the diatom concentration. A DNA-specific dye, SYBR green I 10,000X in dimethyl sulfoxide (Molecular Probes, USA) was diluted by a factor of ten with filtered, sterile seawater (FSSW), divided into aliquots, and then stored at -20°C . This working solution was added at 1% final concentration (v/v) to the second tube, which was used to estimate dinoflagellate as well as bacterial concentrations after 30 min incubation in the dark at room temperature, which allowed the dye to penetrate the cells.

Analyses were done using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a blue laser (argon, 488 nm) and using PBS azide as sheath fluid. Settings of the flow cytometer were adapted to the various cell types (bacteria, diatoms, and dinoflagellates): a 30 second analysis per sample at a low flow rate ($\sim 15 \mu\text{l min}^{-1}$) for bacterial cell count, and 60 second analyses at a high flow rate ($\sim 60 \mu\text{l min}^{-1}$) for diatoms. Due to their lower concentration, dinoflagellates were analyzed at high rate until one hundred cells had been detected. The chlorophyll a red fluorescence of the diatoms was measurable on the FL3 detector ($> 630 \text{ nm}$) of the flow cytometer,

allowing distinction of algal cells from other particles. The SYBR Green fluorescence, related to the DNA content of bacteria and dinoflagellate, was measurable on the FL1 detector (green) of the flow cytometer at 500-530 nm allowing detection of the selected target and calculation of the number of individual cells. Finally, based on the flow rate of the cytometer calculated according to Marie et al. (1999), the total concentration of diatoms, bacteria and dinoflagellates was estimated in cells ml⁻¹.

Rate Calculations: Ingestion rates (I) for *Oxyrrhis marina* and *Strombidium* sp. grazing on diatoms and bacteria were calculated from differences in cell concentrations between control and grazing bottles according to Strom et al. (1997). On few occasions, grazing coefficients determined for a replicate bottle during a specific time interval were negative due to “net growth” of cells in bottles with grazers. For these replicates ingestion was considered zero in the calculation of average rates and standard deviation.

Growth rates (μ ; doublings d⁻¹) of bacteria and microzooplankton were estimated from changes in cell concentrations (C) between two sampling points (t₁ and t₂) assuming exponential growth (Verity and Villareal 1986; Strom et al. 1997).

$$\mu = (\ln C_2 - \ln C_1) * (1/t_2 - t_1)$$

Loss rates and accumulation rates in nmol Si(OH)₄ per liter and day were calculated as

$$\Delta c = (c[\text{Si(OH)}_4]_{t_2} - c[\text{Si(OH)}_4]_{t_1}) / (t_2 - t_1)$$

with c[Si(OH)₄]_{t₁} and c[Si(OH)₄]_{t₂} being the Si(OH)₄ concentrations measured the beginning (t₁) and the end of the time interval (t₂), respectively.

Statistical Tests: Differences of bacterial growth rates, Si(OH)₄ net removal and net release between control and grazing treatments over all three sampling intervals were tested with a repeated measures analysis of variance of ranks (Friedman test). The origin of the differences was determined with the Tukey post-hoc test. Whether microzooplankton grazing increased recycling of Si(OH)₄ compared to the control was tested with Mann-Whitney statistics.

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Tables

Table 1. List of incubation experiments performed in this study. Predator-prey combinations, initial cell abundances and Si(OH)₄ concentrations are presented. Numbers represent the mean of three replicates and the standard deviation from the mean.

Experiment	Treatment	Diatoms (cells ml ⁻¹)	Grazer (cells ml ⁻¹)	Bacteria (cells ml ⁻¹) x 10 ⁶	Si(OH) ₄ (μmol l ⁻¹)
1	<i>Thalassiosira pseudonana</i>	95289 ± 5871		1.80 ± 0.50	2.61 ± 0.07
	<i>T. pseudonana</i> + <i>Oxyrrhis marina</i>	90913 ± 9907	729 ± 220	2.53 ± 0.08	2.90 ± 0.07
2	<i>Thalassiosira pseudonana</i>	99600 ± 12900		0.25 ± 0.03	1.28 ± 0.07
	<i>T. pseudonana</i> + <i>Oxyrrhis marina</i>	100761 ± 8480	827 ± 129	0.89 ± 0.05	4.14 ± 0.05
3	<i>Chaetoceros gracilis</i>	162518 ± 16290		1.14 ± 0.10	2.18 ± 0.15
	<i>C. gracilis</i> + <i>Oxyrrhis marina</i>	159546 ± 4888	442 ± 87	1.72 ± 0.04	1.94 ± 0.25
4	<i>Thalassiosira pseudonana</i>	346943 ± 10544		2.43 ± 0.10	1.46 ± 0.27
	<i>T. pseudonana</i> + <i>Strombidium</i> sp.	327986 ± 13697	47 ± 6	1.96 ± 0.09	1.76 ± 0.06
5	<i>Chaetoceros gracilis</i>	435010 ± 78185		0.39 ± 0.02	1.36 ± 0.10
	<i>C. gracilis</i> + <i>Strombidium</i> sp.	444034 ± 11598	74 ± 19	0.46 ± 0.02	1.24 ± 0.21

Table 2. Results of the statistical analyses to determine significant differences between control and grazing incubations. Friedman test results (χ^2) and probability (p) are presented for differences in bacterial growth rate and changes in Si dynamics. For individual time steps differences between control and grazing treatment are presented based on the Tukey post-hoc test. s = significant, ns = not significant. Symbols in the last column indicate strong (**), weak (*) or no (-) grazing on diatoms during the respective time interval.

Experiment	Friedman		Time interval	Tukey post-hoc		Grazing
	μ Bacteria	Δ [Si(OH) ₄]		μ Bacteria	Δ [Si(OH) ₄]	
<i>T. pseudonana</i> + <i>O. marina</i> (exp 1)	χ^2 =14.76 p=0.01	χ^2 =14.18 p=0.01	0-24 h	s	s	**
			24-48 h	s	ns	*
			48-168 h	s	ns	-
<i>T. pseudonana</i> + <i>O. marina</i> (exp 2)	χ^2 =13.81 p=0.02	χ^2 =13.62 p=0.02	0-24 h	ns	s	**
			24-48 h	s	ns	*
			48-96 h	ns	ns	-
<i>C. gracilis</i> + <i>O. marina</i> (exp 3)	χ^2 =14.19 p=0.01	χ^2 =14.0 p=0.02	0-24 h	s	s	*
			24-48 h	ns	ns	*
			48-168 h	ns	ns	**
<i>T. pseudonana</i> + <i>Strombidium</i> sp. (exp 4)	χ^2 =13.62 p=0.02	χ^2 =10.72 ^{ns} p=0.06	0-24 h	s	s	*
			24-48 h	ns	ns	*
			48-96 h	s	ns	*
<i>C. gracilis</i> + <i>Strombidium</i> sp. (exp 5)	χ^2 =14.38 p=0.01	χ^2 =4.85 ^{ns} p=0.43	0-24 h	ns	ns	-
			24-48 h	ns	ns	-
			48-96 h	ns	ns	-

Table 3. Net change in silicic acid concentrations (mean concentration and standard deviation) for control and grazing treatments of the five experiments. The estimated percentage of bSiO₂ that has been ingested by the grazers, and the probability (p) for Mann-Whitney comparison of median values are indicated for each experiment. n=3

Experiment	Diatom	Treatment	Net change Si(OH) ₄ (μmol l ⁻¹)	% bSiO ₂ ingested	p
1	<i>Thalassiosira</i>	Control	3.5 (0.2)	75.1	0.96
	<i>pseudonana</i>	<i>Oxyrrhis marina</i>	2.1 (0.3)		
2	<i>Thalassiosira</i>	Control	0.3 (0.0)	71.3	0.96
	<i>pseudonana</i>	<i>Oxyrrhis marina</i>	-1.3 (0.0)		
3	<i>Chaetoceros</i>	Control	0.7 (0.2)	3.6	0.04
	<i>gracilis</i>	<i>Oxyrrhis marina</i>	1.7 (0.3)		
4	<i>Thalassiosira</i>	Control	-0.1 (0.2)	0.02	0.96
	<i>pseudonana</i>	<i>Strombidium</i> sp.	-0.4 (0.1)		
5	<i>Chaetoceros</i>	Control	-0.2 (0.1)	0.6	0.33
	<i>gracilis</i>	<i>Strombidium</i> sp.	-0.1 (0.3)		

Table 4. Change in silicic acid concentration and ingestion rate of diatoms by the grazer over 24h of the incubation. Experiments 1-5: this study; experiments A, B: S. Schultes unpubl. data. Probability (p) of the Mann-Whitney comparison of median values is indicated for each experiment. n=3 (exp 1-5), n=4 (exp A, B). Numbers represent the mean and the standard deviation of the mean.

Expt	Treatment	$\Delta [\text{Si(OH)}_4] \text{ nmol l}^{-1} \text{ d}^{-1}$		p	Ingestion rate cells ind ⁻¹ d ⁻¹
		control	grazing		
1	<i>T. pseudonana</i> + <i>O. marina</i>	-43 (119)	-400 (217)	0.04	27 (20)
2	<i>T. pseudonana</i> + <i>O. marina</i>	177 (91)	-2153 (182)	0.04	34 (14)
3	<i>C. gracilis</i> + <i>O. marina</i>	-537 (136)	-86 (359)	0.81	4 (4)
4	<i>T. pseudonana</i> + <i>Strombidium</i> sp.	-110 (255)	-633 (15)	0.04	25 (24)
5	<i>C. gracilis</i> + <i>Strombidium</i> sp.	-95 (247)	-247 (136)	0.39	no grazing
A	<i>T. weissflogii</i> + <i>A. clausii</i>	-632 (729)	-3038 (158)	0.02	1309 (223)
B	<i>T. punctigera</i> + <i>A. clausii</i>	-2093 (922)	-2427 (224)	0.56	42 (24)

Figure legends

Figure 1: Example of changes in the abundance of diatoms (circles), the micrograzer (squares; both in panel A) and bacteria (panel B) in the grazing (open symbols) and control (full symbols) incubations. Values represent the mean of three replicates; error bars indicate the standard deviation.

Figure 2: Grazing of *Oxyrrhis marina* on a culture of *Thalassiosira pseudonana* (Exp I). Ingestion of diatom cells by the dinoflagellate (A), growth of the dinoflagellate (B), growth of bacteria (C) and changes in silicic acid concentrations (D) are presented. Values represent the mean of three replicates of the control (black) and the grazing treatment (grey). Error bars indicate the standard deviation.

Figure 3: Grazing of *Oxyrrhis marina* on a culture of *Thalassiosira pseudonana* (Exp II). For legend see Fig. 2

Figure 4: Grazing of *Oxyrrhis marina* on a culture of *Chaetoceros gracilis* (Exp III). For legend see Fig. 2

Figure 4: Grazing of *Strombidium* on a culture of *Thalassiosira pseudonana* (Exp IV). For legend see Fig. 2

Figure 6: Grazing of *Strombidium* on a culture of *Chaetoceros gracilis* (Exp V). For legend see Fig. 2

Figure 7: Changes in bSiO_2 concentrations over three time intervals in experiment 2. Mean values for three replicates and the standard deviation are present for the control (black bars) and grazing treatment (grey bars).

Figure 8: Schematic representation of the grazing and microbial processes involved in Si cycling.

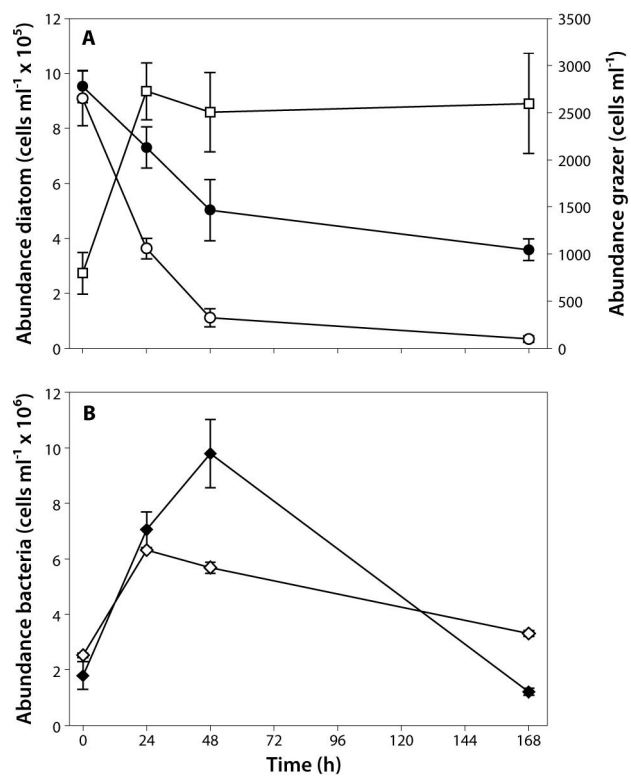
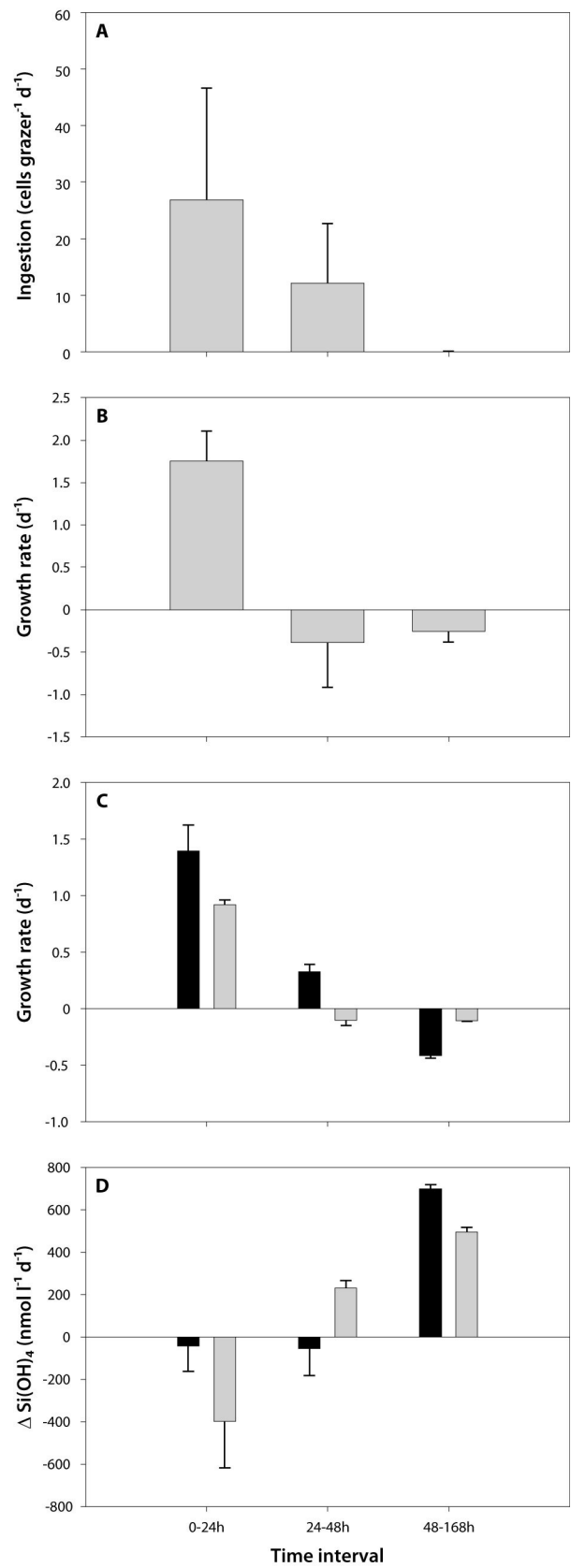


Figure 1:

Figure 2:



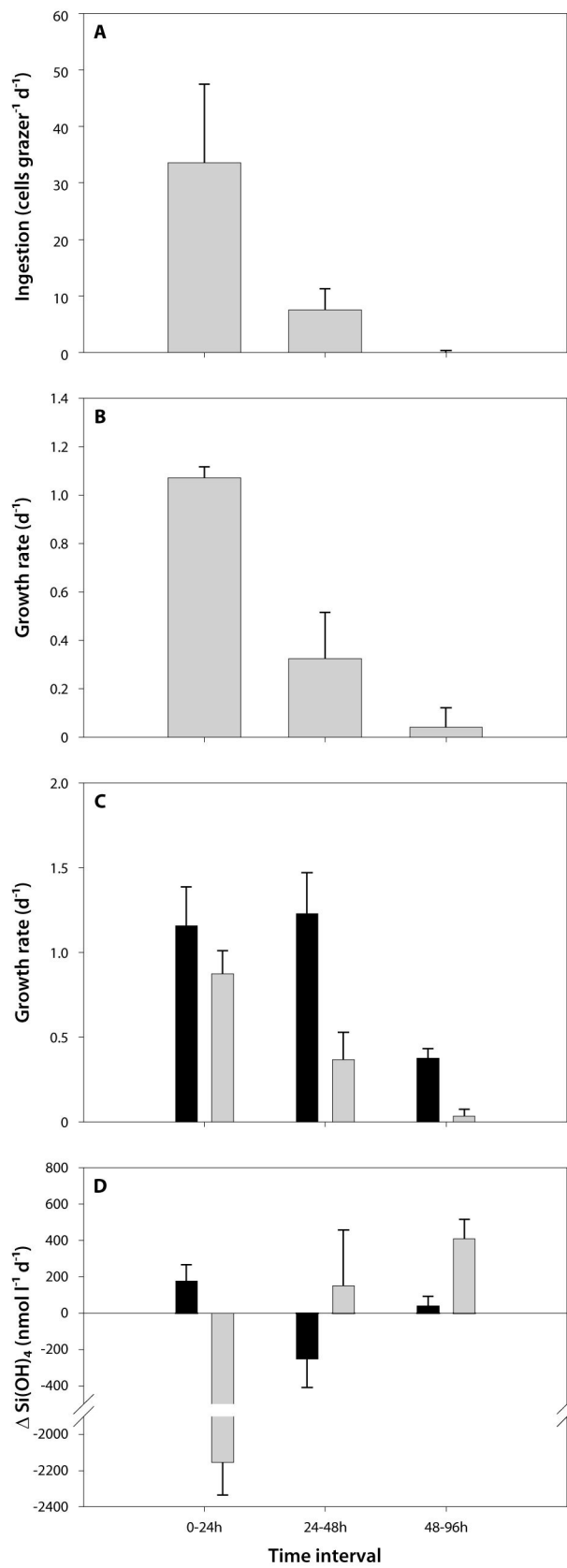


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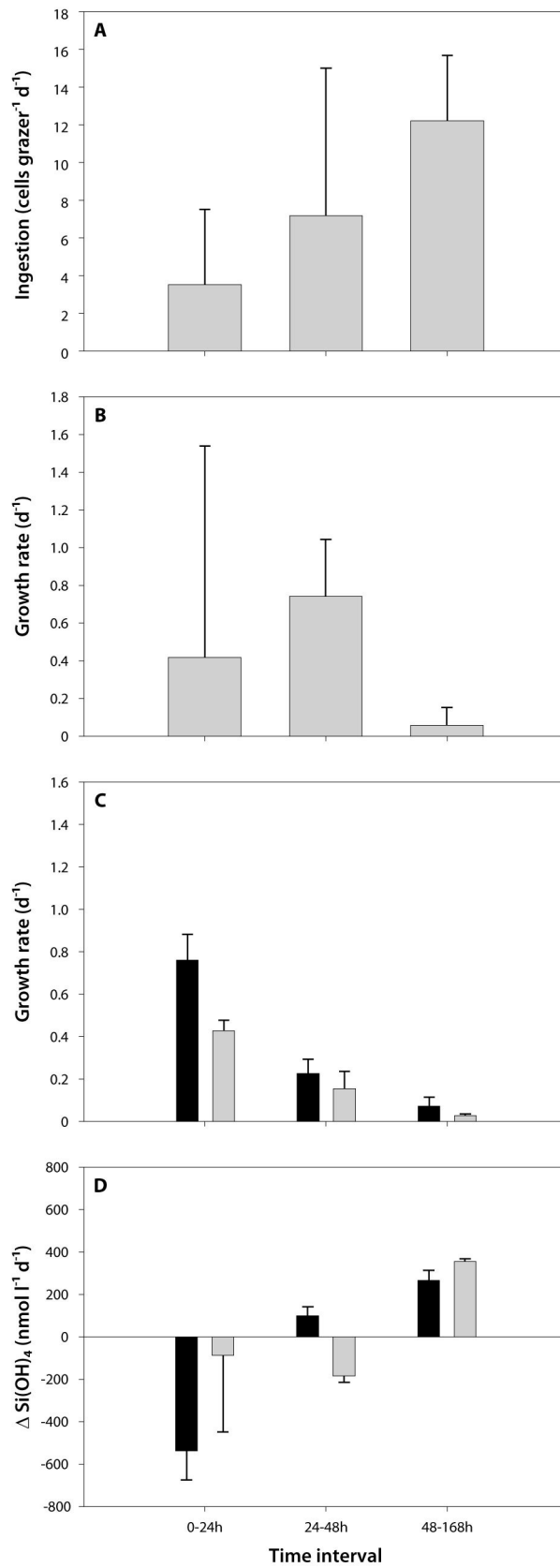


Figure 4 :

Figure 5 :

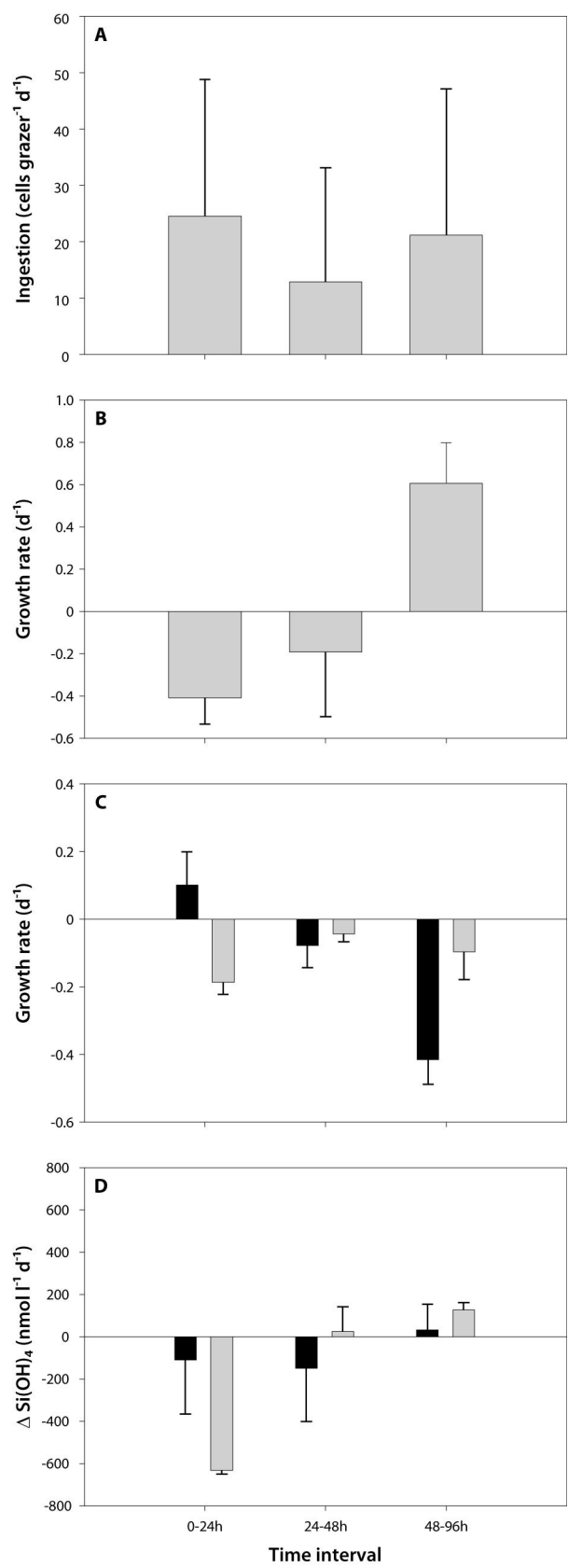
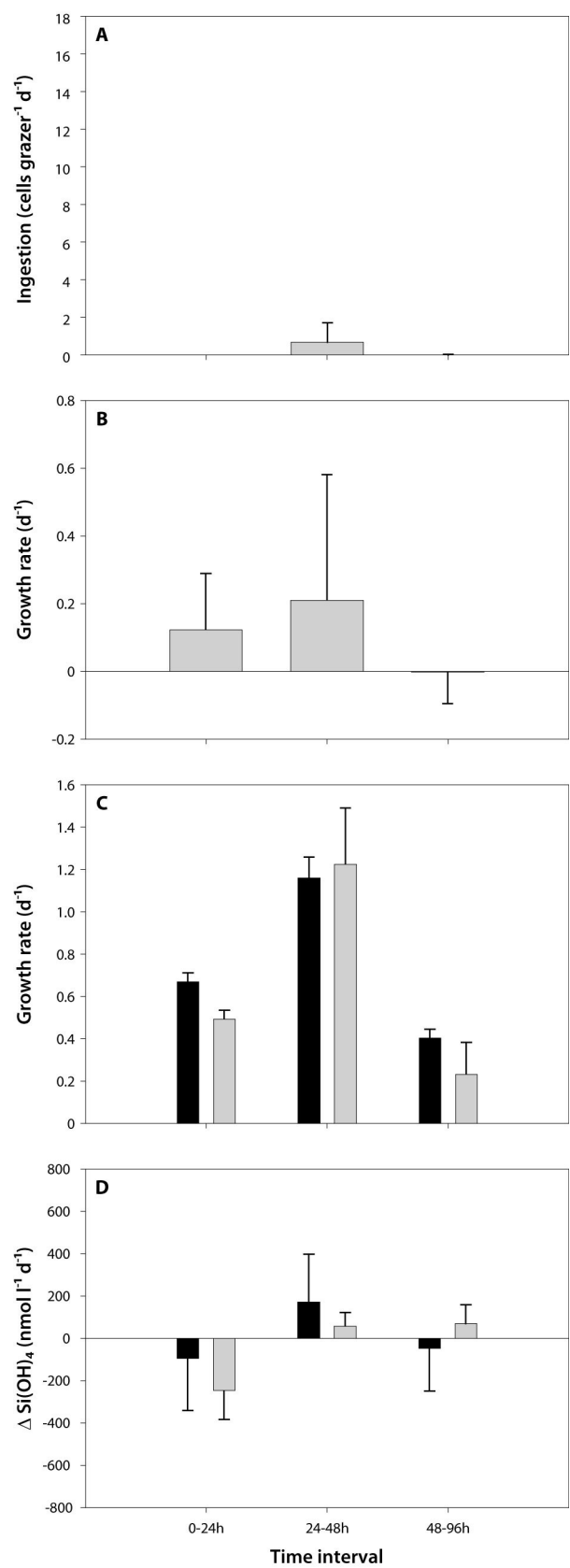


Figure 6 :



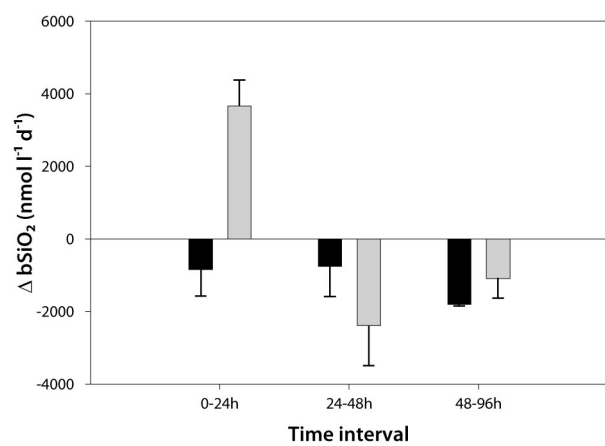


Figure 7:

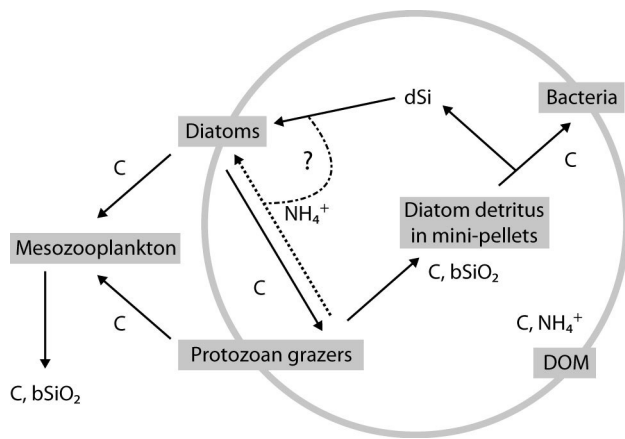


Figure 8: